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Absorption of Tricarballylic Acid from the Rumen of Sheep and Cattle Fed Forages Containing *trans*-Aconitic Acid

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ABSTRACT

Some forages accumulate high concentrations (<5% of dry matter) of trans-*aconitate, and this acid has been implicated in Mg chelation and the occurrence of grass tetany in ruminants.* In vitro *experiments have indicated that rumen microorganisms convert* trans-*aconitate to tricarballylate. The feeding studies described here were conducted to demonstrate absorption of tricarballylate by ruminant animals fed diets similar to those producing grass tetany. When sheep were switched from a diet containing alfalfa (lucerne) (*Medicago sativa *L.) hay (no detectable* trans-*aconitate) to wheat (*Triticum aestivum *L.) and rye (*Secale cereale *L.) forage containing 1·52 and 1·37%* trans-*aconitate, respectively, there was a rapid increase in blood plasma tricarballylate.* Trans-*aconitate was not detected in the plasma. At 16 h after feeding, plasma tricarballylate concentrations were 0·58±0·08 and 0·48±0·21 mM in sheep fed the wheat and rye forage, respectively. Tricarballylate concentrations remained relatively constant for the remaining 60 h of the experiment. Cattle were fed rye forage one week later, and the concentration of* trans-*aconitate in the forage had dropped to 0·83% of the dry matter. Once again there was a rapid*

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appearance of tricarballylate in plasma, but the maximum concentration was 0·31±0·05 mM (t=27 h). When the cattle were removed from the rye forage, there was a linear decline in tricarballylate and none was detected 24 h later. The studies indicated that trans-*aconitate is converted to tricarballylate in the rumen and that tricarballylate rather than* trans-*aconitate is absorbed.*

Key words: Tricarballylate, aconitate, magnesium, grass tetany.

1 INTRODUCTION

Magnesium deficiency of ruminants has been recognized since the 1930s and the symptoms have been identified by a variety of names including grass tetany, lactation tetany, and grass staggers. The disease is most often observed in lactating cattle and sheep grazing succulent, cool-season grass species.[1-4] The availability of forage Mg is influenced by a number of factors including low concentrations of nonstructural carbohydrates and high concentrations of K, N, higher fatty acids, and other organic acids.[5] The finding of large amounts of *trans*-aconitate in forages known to induce grass tetany has led several researchers[6-8] to suggest that *trans*-aconitate might chelate Mg and reduce the availability of dietary Mg. Oral administration of *trans*-aconitate or citrate in conjunction with KCl induced grass tetany symptoms that were corrected by Ca and Mg therapy.[9] The symptoms did not occur when the animals were given orally either acid independent of the KCl. In another study, *trans*-aconitate disappeared rapidly from the sheep rumen, but little *trans*-aconitate was detected in either blood or urine.[10]

In vitro experiments indicated that *trans*-aconitate was rapidly fermented by rumen microorganisms.[11] Some of the *trans*-aconitate was converted to acetate but as much as 50% was converted to tricarballylate (Fig. 1). Tricarballylate was a fermentation product of *trans*-aconitate but not of other organic acids *in vitro*. Tricarballylate was not metabolized further by rumen microorganisms, and it appeared that tricarballylate rather than *trans*-aconitate would remain in the rumen for a long enough time to affect Mg absorption.[11] Sheep given gelatin capsules filled with *trans*-aconitate absorbed significant amounts of tricarballylate, not aconitate, into blood.[12] *In vitro* studies with purified enzymes and isolated hepatocytes indicated that tricarballylate was an inhibitor of the enzyme aconitase and could inhibit acetate oxidation in the citric acid cycle.[12]

The formation of tricarballylate was previously verified with animals fed timothy hay (*Phleum pratense* L.).[11,12] Since this forage did not contain detectable amounts of *trans*-aconitate, reagent-grade *trans*-aconitate was provided as a supplement. The following experiments were performed in the western United States where *trans*-aconitate-accumulating plants are the predominant range forages.[7] Feeding trials were initiated during the early spring when lush grasses usually contain the highest concentration of *trans*-aconitate.

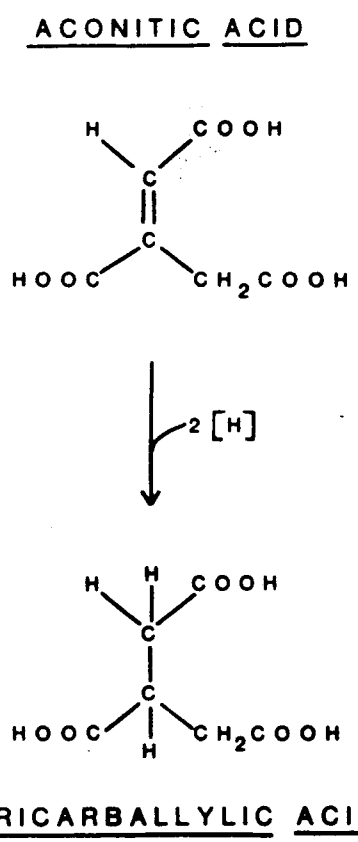

Fig. 1. Structure and likely pathway of aconitate conversion to tricarballylate. Taken from reference 11 with permission.

2 EXPERIMENTAL

2.1 Feeding trials

Six nonlactating, nonpregnant, 6-year-old white-face ewes (56 kg) and two lactating 2-year-old Suffolk ewes (61 kg), each with twin lambs (24 kg), were fed alfalfa (*Medicago sativa* L.) at 0500 and 1600 for 4 days prior to diet change. The ewes were randomly assigned to one of two treatment groups with one lactating ewe in each group. One group received freshly harvested wheat (*Triticum aestivum* L.) forage (Table 1) which was in the preboot stage of growth (23% dry matter), and the other group was given rye (*Secale cereale* L.) in the early preboot stage (19% dry matter). The forage was fed at 0500 and 1600 for the duration of the experiment, and water was provided *ad libitum*. A second experiment was conducted with four lactating beef cows weighing approximately 410 kg. These animals were also fed alfalfa hay for 4 days before the change in diet. During the experimental period (1·5 days), cows were fed (0500 and 1600) freshly harvested rye forage.

TABLE 1
Concentrations of *trans*-Aconitate, Sugars and Minerals in Forages Fed to Sheep and Cattle

Animal	*Forage*	*Forage composition*										
		Aconitate	*Glucose*	*Fructose*	*Sucrose*	*Ca*	*Mg*	*K*	$\frac{K}{Ca+Mg}$	*Ash*[a] *alkalinity* (*meq* kg^{-1})	*Dry matter*[b] *intake* (*kg*)	*Aconitate*[b] *intake* (*mM*)
		(% dry matter)										
Ewes	Alfalfa hay	0·00	2·30	0·70	0·80	1·29	0·32	2·00	0·56	0	*ad lib.*	—
	Rye	1·37	1·40	1·28	11·19	0·31	0·13	2·86	2·80	626	4·1	330
	Wheat	1·52	1·24	1·10	14·21	0·31	0·13	2·92	2·85	629	4·6	410
Cows	Alfalfa hay	0·00	1·10	0·60	1·10	1·27	0·34	2·38	0·66	0	*ad lib.*	—
	Rye	0·83	1·40	1·02	11·95	0·28	0·13	2·89	3·00	640	12·5	610

[a]Residual ash corrected for nitrate loss which is a good estimate of total organic acids.[20]
[b]Intake for the experimental feeding period of 76 and 32 h for ewes and cows, respectively.

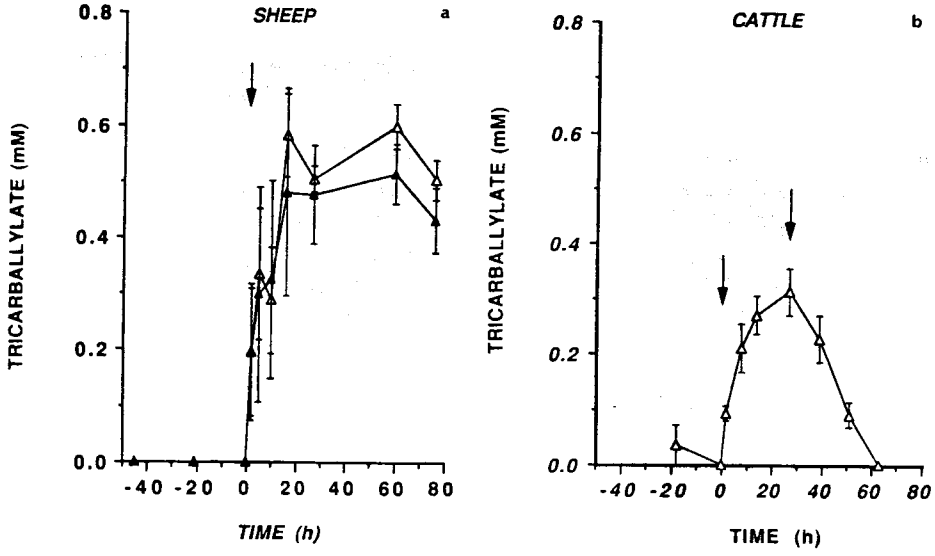

Fig. 2. Effect of forage (△, wheat, or ▲, rye) on the concentration of tricarballylate in the blood of sheep (a) and cattle (b). The animals were switched from alfalfa to rye or wheat at time 0 (⬌). The cattle were switched back to lucerne at 24 h (⬌).

2.2 Analyses

Blood was drawn by jugular puncture into heparinized tubes (see Fig. 2 for interval). The blood was centrifuged, and the plasma was frozen and preserved during transfer from Kimberly, Idaho, to Ithaca, New York, for analysis. Representative forage samples were dried at 60°C for 48 h to calculate dry matter content, and the samples were ground to pass through a 1-mm sieve.

Forage samples were extracted with 0·026 M H_2SO_4 (24 h at 5°C) and centrifuged (10000×g, 15 min, 0°C) to remove insoluble forage residue. The forage extract and blood samples were deproteinized with $HClO_4$ (0·5 M, final concentration) and the ClO_4^- was removed with an excess of K_2CO_3. Organic acids and sugars were measured by high-pressure liquid chromatography using a Beckman model 334 liquid chromatograph that was equipped with a model 156 refractive index detector and a Bio Rad HPX-87H organic acid column.[13] The sample size was 50 μl, the eluent was 0·026 M H_2SO_4, and the column temperature was 50°C. Magnesium, Ca and K were measured by atomic absorption spectrophotometry.[14]

3 RESULTS AND DISCUSSION

During the background period the sheep were fed alfalfa hay that did not contain any detectable aconitate (Table 1). At time zero the sheep were given either wheat or rye forage, and the intake of *trans*-aconitate was 410 and 330 mM, respectively, over the next 72 h. Soon after feeding there was a rapid increase in

blood tricarballylate (Fig. 2a), and aconitate was not detected in any of the blood samples. Within 2 h tricarballylate concentrations in plasma had reached 0·2 mM, and by 16 h the concentration was 0·58 mM with the wheat and 0·48 mM for the rye forage. During the next 60 h, tricarballylate ranged from 0·44 to 0·60 mM. Tricarballylate absorption did not affect ($P<0·05$) plasma concentrations of Mg or Ca, and tetany was not observed. During the background period, the concentrations of plasma Mg and Ca ($n=16$) were 24·8±0·4 and 113·4±1·4 mg liter^{-1}, respectively. After the change in diet, the values ($n=56$) were 24·8±0·4 and 115±1·4 mg liter, respectively. In this study the intake of *trans*-aconitate was probably lower than amounts consumed by many animals that have developed grass tetany. Grasses sometimes contain more than 5% *trans*-aconitate.[6]

The cattle study was conducted one week after the sheep study, and the aconitate content of the rye forage had declined to 0·83% (Table 1). When the cattle were switched from alfalfa to rye, plasma concentrations of tricarballylate increased rapidly (Fig. 2b). There was only 60% as much aconitate in the rye during this trial as in the previous trial, and the maximum concentration of tricarballylate in plasma was half that observed in the sheep trial (Fig. 2). When the cattle were switched back to alfalfa hay, blood concentrations of tricarballylate declined rapidly. While being fed the rye diet, the cows ingested 610 mM *trans*-aconitate. Plasma Mg and Ca were 24·4±0·9 and 117·6±2·7 mg liter^{-1}, respectively, during the background period and did not change significantly after tricarballylate was absorbed (23·0±0·09 and 122±1·2, respectively).

Previous studies indicated that *trans*-aconitate was rapidly metabolized to tricarballylate.[11,12] The almost immediate appearance of tricarballylate in the plasma of sheep and cattle in the study reported here is consistent with the earlier observations. The forages used in this study were not particularly high in *trans*-aconitate; however, plasma concentrations of tricarballylate were greater than 0·5 mM in the sheep study (Fig. 2a). Since the plasma concentration seemed to be dose dependent (sheep versus cattle trial), it is conceivable that plasma concentrations could be greater in ruminants grazing forage with even more *trans*-aconitate.

The wheat and rye forages contained high concentrations of sucrose in addition to *trans*-aconitate (Table 1), and the sugar may have enhanced tricarballylate formation. When rumen bacteria capable of reducing aconitate to tricarballylate were isolated, *Selenomonas ruminantium* was the predominant species.[15] *S. ruminantium* has an extremely high affinity for sucrose ($K_m=4$ μM), and its numbers increase greatly when sugars are present in the rumen.[16,17] Bacteria were not enumerated in this study, but, on the basis of sucrose content in the wheat and rye forage, the numbers of aconitate-reducing bacteria should have increased during the experimental period.

When cattle were returned to the alfalfa diet, tricarballylate concentrations in the blood declined (Fig. 2b). Disappearance could have been attributed to either metabolism or excretion, but the latter seemed more likely. Tricarballylate is an effective metabolic inhibitor and it is not rapidly metabolized by mammalian enzymes.[12] Renal clearance rates[18] could account for the disappearance of tricarballylate.

4 CONCLUSIONS

These experiments provide evidence that sheep and cattle fed forages containing *trans*-aconitate converted the *trans*-aconitate to tricarballylate, another acid capable of chelating magnesium (stability constant of 115).[19] Since tricarballylate rather than *trans*-aconitate was absorbed, tricarballylate could be a significant factor in the etiology of 'grass tetany'. Further experiments should be conducted to ascertain blood concentrations of tricarballylate in tetanic animals, the ability of tricarballylate to inhibit the citric acid cycle, and the capacity of tricarballylate to increase urinary losses of magnesium.

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